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**The effect of Vancomycin and Gentamicin antibiotics on human osteoblast proliferation, metabolic function and bone mineralisation**

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**Structured Abstract**

**Study Design:** This study investigates the effect of Vancomycin and Gentamicin antibiotics on primary human osteoblasts. Osteoblasts were incubated with vancomycin, gentamicin or with Povidone-Iodine (PVI), at concentrations advocated for wound irrigation. Osteoblast proliferation, metabolic function and bone mineralisation were measured.

**Objective:** To model Gentamicin and Vancomycin wound irrigation *in vitro* and examine the effect on osteoblast viability and cellular function in comparison to 0.35% PVI.

**Summary of Background Data:** Vancomycin, Gentamicin and dilute PVI are employed as wound irrigants in spinal surgery to reduce infection. However, we have recently demonstrated that 0.35% PVI has a detrimental effect on osteoblast cellular function and bone mineralisation. Studies to determine the effects of antibiotic wound irrigation solutions on osteoblasts and bone mineralisation are therefore warranted.

**Methods:** Primary human osteoblasts were exposed for 20 min to either PBS control, Vancomycin (35 mM or 3.5 mM), Gentamicin (34 mM or 3.4 mM) or 0.35% PVI for 3 min. Cellular proliferation was measured over 7 days by MTS assay. Osteoblast metabolic function was determined using a Seahorse XFe24 Bioanalyzer. Mineralised bone nodules were quantified using Alizarin red.

**Results:** At concentrations advocated for wound irrigation, both Gentamicin (3.4 mM) and Vancomycin (3.5 mM) induced a transient 15-20% reduction in osteoblast proliferation, which returned to control values within 72 h. This was in marked contrast to the effect of 0.35% PVI which resulted in a sustained reduction in osteoblast proliferation of between 40-50% over 7 days. Neither Gentamicin nor Vancomycin at concentrations up to 10x clinical dose had any effect on osteoblast oxygen consumption rate, or significantly affected mineralised bone nodule formation.

**Conclusion:** Vancomycin and Gentamicin solutions, at concentrations advocated for intrawound application in spinal surgery have a small but transient effect on osteoblast proliferation, and no effect on either osteoblast metabolic function or bone nodule mineralisation.

### **Key Words**

Adolescent idiopathic scoliosis; osteoblasts; vancomycin; gentamicin; povidone iodine; PVI; bone; wound irrigation; antibiotics; bone mineralisation; cell proliferation, cell metabolism, surgical site infections

Level of Evidence: N/A

## **Key Points**

- Acute exposure to clinically employed concentrations of Gentamicin and Vancomycin (3.4 mM and 3.5 mM respectively) induces a small but transient reduction in the proliferation of osteoblasts, in contrast to 0.35% PVI which induces a marked and sustained reduction in osteoblast proliferation
- Gentamicin or Vancomycin up to 10x clinical dose did not affect osteoblast metabolic capacity (OCR), or effect bone nodule mineralisation, in contrast to 0.35% PVI.

### **Mini abstract**

This study modelled the effect of using Gentamicin and Vancomycin antibiotics during spinal surgery on human primary osteoblast cells. Osteoblasts treated with clinically employed concentrations of Gentamicin or Vancomycin, exhibited a small but transient reduction in proliferation, and no change in metabolic capacity or ability to form mineralised bone.

## 1 Introduction

2 Surgical site infections (SSIs) following spinal surgery are a serious complication,  
3 which negatively impact on patient outcomes and can be fatal. Such incidences can  
4 require additional operations involving antibiotic irrigation of the wound and  
5 debridement <sup>1,2</sup>, and in certain cases, the spinal implants must be removed: an  
6 expenditure of both time and healthcare resources <sup>3</sup>. Indeed, infection-related costs  
7 have been reported to be an additional \$30,000 per patient <sup>4</sup>.

8  
9 In order to combat infection, several prophylactic measures are in use <sup>5</sup>. Peri-  
10 operative broad spectrum antibiotic usage is standard in orthopaedic surgery and  
11 does significantly reduce risk of infection <sup>6</sup>. However, in addition, in order to reduce  
12 rates further, and due to the rise of Methicillin-Resistant *Staphylococcus Aureus*  
13 (MRSA) <sup>7</sup>, intrawound broad-spectrum antibiotics including Vancomycin and  
14 Gentamicin <sup>8,9,10</sup> may be applied. Unfortunately, there are very few  
15 recommendations from historical literature with regards to the use of antibiotics to  
16 combat SSI rates in spinal surgery, and thus clinical practice varies considerably.

17  
18 In a systematic review, particularly high SSI rates (3.5% to 8.5%) were noted in  
19 spinal surgery in paediatric patients with cerebral palsy, spina bifida, patients with  
20 urinary tract infections, incontinence obesity and those patients undergoing pelvic  
21 fixation procedures <sup>11</sup>. This review was the basis for constructing the Best Practice  
22 Guidelines, where the intrawound application of Vancomycin is specifically  
23 recommended <sup>12</sup> for spinal surgery in paediatric patients. Several studies, including  
24 large randomised control trials, have provided evidence that administration of  
25 intrawound Vancomycin is safe <sup>13</sup> and efficacious in reducing the incidence of SSIs,

when compared to prophylactic antibiotics alone <sup>14,15,16,17,18</sup>. Similarly, intrawound application of Gentamicin has also been shown to reduce infection rates spinal surgery in children with cerebral palsy, with a reduction from 15.2% to 3.9% observed <sup>19</sup>.

Some spinal units have reported that wound irrigation with a solution of Povidone-Iodine (PVI;Betadine) <sup>20,21</sup> is safe and efficacious at reducing SSI rates in spinal surgery. Furthermore, a protocol which combined PVI wound irrigation with intrawound Vancomycin powder was recently advocated as being highly effective at reducing SSI rates in spinal surgery, with a reduction of 50% reported <sup>22</sup>. However, we have recently reported that exposure of human osteoblasts to 0.35% PVI (the concentration advocated for wound irrigation), is highly cytotoxic to osteoblasts, with a marked and sustained reduction in osteoblast cellular proliferation and inhibition of mineralised bone formation <sup>23</sup>. This particular study highlighted the importance for spinal units to evaluate not only the SSI antibacterial efficacy of such intrawound reagents, but to also to consider the effect of these reagents on the cellular osteoblast function.

Critically, no studies have reported the effects of Vancomycin or Gentamicin antibiotics on human primary osteoblast mineralised bone formation. Neither has their effect on osteoblast proliferation been reported following an *in vitro* protocol that mimics the transient high concentrations of antibiotics in the surgical wound following intrawound application. Given that the goal of many spinal surgeries is to establish solid bone union, it is important to know whether these reagents, like PVI, may impair bone healing since this may help guide clinical practice. The aim of this study



51 was therefore to model *in vitro* the effect of Vancomycin and Gentamicin antibiotic  
52 solutions on osteoblast proliferation, osteoblast metabolism and mineralised bone  
53 nodule formation.

54

## METHODS

### 3.1 Cell culture

Ethical approval was granted by the United Kingdom (UK) National Research Ethics Service (National Health Authority, reference NRES 14-ES-1044), and institutionally approved and sponsored by the University of Birmingham as required under the UK Research Governance Framework. Study participants were provided in advance with a participant information sheet, and a participant consent form.

Following patient's written consent, the femoral head was collected from a female patient (aged 62 years) undergoing total joint replacement surgery for hip osteoarthritis. The articular cartilage was removed from the femoral head and the subchondral bone cut into small chips. The bone chips were then washed thoroughly in serum-free primary osteoblast media to remove any excess blood, connective or adipose tissue and then incubated in differentiation media (10% FCS, penicillin (100units/ml), streptomycin (100µg/ml), L-glutamine (2mM), β-glycerophosphate (2mM), Ascorbic acid (50µg/ml) and Dexamethasone (10nM)) in a culture flask at 37°C (5% CO<sub>2</sub>). Differentiation media was replaced with fresh media 2x per week, and the bone chips removed upon the appearance of osteoblast cells. Primary human osteoblast cells were cultured in differentiation media.

The experimental concentrations of Vancomycin and Gentamicin utilised for this study were based on the serial observation of average wound drain outputs following turning patients from the table. From this it was estimated that an average scoliosis wound held approximately 200 ml of fluid. Furthermore, it was considered that the majority of Vancomycin and Gentamicin antibiotic would be washed out after closure

following opening of the drain after 20 min. Therefore an experimental concentration of 1g of Vancomycin or Gentamicin in 200ml of PBS solution (equating to 3.5 mM and 3.4 mM respectively) exposed for 20 min would best mimic the clinical concentration and exposure time. Higher concentrations at 10X the estimated clinical concentration was utilised for the second experimental setting, equating to 35 mM and 34 mM for Vancomycin and Gentamicin respectively. The experimental concentration of PVI utilised was 0.35%, which has previously been clinically advocated. Cells were exposed for 3 min with 0.35% PVI, before washing with PBS in order to mimic the clinical saline washout procedure.

### 3.3 Osteoblast proliferation assay

Primary human osteoblasts were seeded at  $6 \times 10^3$  cells per well in a 96 well plate. At confluency, osteoblasts were stimulated with either Vancomycin (3.5 mM or 35 mM), or Gentamicin (3.4 mM or 34 mM) for 20 min, or with 0.35% PVI for 3 min. All wells were then aspirated, washed 5x with PBS and then filled into osteoblast differentiation media. After 0, 24, 48, 72 and 144 h incubation at 33°C, an MTS (Cell Titer Aqueous One Solution Cell Proliferation Assay, Promega) assay was performed as per manufacturer's instructions as a measure of osteoblast proliferation.

### 3.4 Osteoblast metabolic function

Primary human osteoblasts were plated at  $6 \times 10^3$  cells per well in a XF<sup>e</sup> 24 Cell Culture Microplate (Seahorse Bioscience, USA). At confluency, the cells were stimulated, washed and incubated exactly as in the proliferation assay and then

placed back into media. After 24 h incubation at 33°C, XF Assay medium (XF base medium with 2mM GlutaMAX™) was then added to the plate and incubated at 33°C for 1 h. Osteoblast oxygen consumption rate (OCR) was determined using a XF<sup>e</sup> Extracellular Flux Analyzer (Seahorse Bioscience, USA), as a measure of osteoblast metabolic function.

### 3.7 Osteoblast bone nodule formation and mineralization assay

Primary human osteoblasts were seeded at  $6 \times 10^3$  cells per well in an 96-well plate and stimulated, incubated and washed as previously described before being cultured in differentiation media. After 28 days, cells were stained with Alizarin red solution in order to quantify the degree of mineralisation following the formation of bone nodules. Briefly, cells were incubated in 0.5% Alizarin Red staining solution (Sigma-Aldrich, UK) in 1% ammonia solution at pH 4.5 for 10min at room temperature and washed with PBS to remove excess stain. Cells were then incubated in 10% cetyl pyridinium chloride (Sigma-Aldrich, UK) for 10 min at room temperature. The supernatant was collected from each well and diluted 1:10 with the 10% cetyl pyridinium chloride and read at OD<sub>550nm</sub> on a SpectraMAX Microplate Reader (Molecular Devices, USA).

### 3.8 Statistical Analysis

All statistical analyses were carried out using SPSS. Unless otherwise stated, all data within figures represents Mean  $\pm$ SEM and were assessed by a two-way analysis of variance (ANOVA) with a Bonferroni post-hoc test conducted where appropriate. Significance was accepted as  $P < 0.05$ .

## Results

### 3.1 The effect of acute exposure to Vancomycin and Gentamicin antibiotics on osteoblast proliferative activity

Relative to normal osteoblast proliferation (PBS control), 20 min exposure of human osteoblasts to Gentamicin at the clinical concentration of 3.4 mM resulted in a significant reduction in osteoblast proliferation of 12% after 24h ( $P<0.05$ ), and 22% after 48 h ( $P<0.01$ ). However, at 3 days, osteoblast proliferation had returned to control levels. At 10x clinical dose of 34 mM Gentamicin, a similar reduction in osteoblast proliferation was observed, peaking at a 27% reduction after 48 h ( $P<0.001$ ). However, osteoblasts exposed to 34 mM Gentamicin exhibited a more sustained reduction in osteoblast proliferation, with a significant 14% reduction ( $P<0.05$ ) observed at 7 days post exposure (Figure 1A).

Vancomycin at 3.5 mM had no significant effect on osteoblast proliferation at 24 h, but there was a small 15% reduction in proliferation relative to control at 48 h. However, proliferation levels returned to control levels at 3 days post exposure. Similar to our findings with Gentamicin, the 10x clinical dose of 35 mM Vancomycin induced a more pronounced inhibition of osteoblast proliferation of between 18-24% and this effect was sustained up to 7 days post exposure (Figure 1B).

In contrast, osteoblasts exposed to 0.35% PVI for 3 min exhibited a much greater reduction in proliferation of between 40-50% which was sustained between 24 h and 7 days ( $P<0.001$ ) (Figure 1A & 1B).

### 3.2 Acute exposure to Vancomycin and Gentamicin antibiotics does not affect human osteoblast basal metabolic function.

Given these differential findings between the effects of antibiotic reagents and PVI on osteoblast proliferation, we next compared their effects on osteoblast metabolic function. As we have previously reported, osteoblasts exposed for 3 min to 0.35% exhibit a significant 94% reduction ( $P < 0.001$ ) in basal oxygen consumption rate (OCR)<sup>23</sup>. However, in contrast neither Gentamicin nor Vancomycin, at either clinical dose or 10x clinical dose, elicited any significant reduction in osteoblast OCR. In osteoblasts that had been exposed 35 mM Vancomycin, OCR was on average lower than PBS control, although this did not reach statistical significance (Figure 2A & 2B).

### 3.4 Acute exposure of human osteoblasts to Vancomycin and Gentamicin does not inhibit the formation of mineralised bone

We next examined whether acute 20 min exposure of human osteoblasts to Vancomycin or Gentamicin had a chronic effect on their ability to form mineralised bone nodules. Following exposure to Vancomycin, Gentamicin or PBS control, confluent osteoblasts were kept in culture for a further 2 weeks before mineralised bone nodules were stained and quantified using Alizarin red (Figure 3A). Although, on average the degree of bone mineralisation was lower in osteoblasts which had been exposed to antibiotics, there was no significant reduction observed with either Gentamicin or Vancomycin at either clinical dose or 10x clinical dose (Figure 3B and 3C).

## Discussion

This study shows that, at concentrations advocated for intrawound application, Vancomycin (3.5 mM) and Gentamicin (3.4 mM) elicit a small (15-20% reduction) in osteoblast proliferation. Critically however, this effect was transient, with osteoblast proliferation returning to normal within 72 h post-exposure. Furthermore, there was no impact on either osteoblast metabolic capacity or the ability of osteoblasts to form mineralised bone at these concentrations. These findings were in stark contrast to our findings with PVI, where the detrimental effects on osteoblast proliferation were significantly greater (40%-50% inhibition) and were sustained for up to 7 days.

These findings are clinically important since there are clear advantages to surgical intrawound application of antibiotics compared to intravenous administration. Firstly, in the Best Practice Guidelines, intrawound application of Vancomycin is favoured over intravenous delivery due to concerns about its potential systemic toxicity <sup>12</sup>. Critically, although intrawound vancomycin is absorbed systemically, the systemic levels are low <sup>14</sup>, thus higher concentrations can be applied where required without concerns for systemic toxicity <sup>14</sup>. Furthermore, wounds are complicated by seroma, haematomas and devitalised tissue. Such tissues present barriers, which systemic prophylactic antibiotics poorly penetrate <sup>24</sup>. Indeed, in a rabbit surgical wound infection model, intrawound Vancomycin was significantly more effective at eliminating SSIs than intravenous cefazolin alone <sup>25</sup>.

Despite our data showing that Vancomycin (at 3.5 mM) and Gentamicin (at 3.4 mM) elicit only a small and transient effect on osteoblast proliferation, a previous study using the human MG-63 osteosarcoma cell line <sup>26</sup> found that Vancomycin at 1000

ug/ml (equivalent to 0.68 mM) had no effect on cell viability, but was toxic at a concentration of 10,000 ug/ml (6.8 mM). Similarly, Gentamicin at concentrations up to 1000 ug/ml (2 mM) have previously been reported to reduce alkaline phosphatase activity in osteoblast-like cells from cancellous hip bone <sup>27</sup>. However, it is important to note that firstly in the study by Edin *et al.* <sup>26</sup> the utilised cells were not primary cells and, although classed as “osteoblast-like”, their proliferation rate and alkaline phosphatase activity is not considered very representative of bone <sup>28</sup>. Secondly, in the study by Edin *et al.* <sup>26</sup> the cells were exposed to Vancomycin for 24-72 hours, and in the study by Isefuku *et al.* <sup>27</sup> cells were exposed to Gentamicin for 4 days. Such lengthy *in vitro* protocols are likely to poorly mimic the transient high concentration of Vancomycin within the surgical site following intrawound clinical application <sup>14</sup>. Our *in vitro* data supports clinical observations which report no indications of bone growth problems in patients where bone allografts have been supplemented with Vancomycin in hip revision surgery <sup>29</sup>.

Importantly, despite the Best Practice Publication <sup>12</sup> not including PVI irrigation amongst the recommendations, the use of PVI either alone or in combination with intrawound antibiotics is still being advocated by several spinal units <sup>22,20,21</sup>. Furthermore, there are currently no clinical studies which have demonstrated greater gains in reducing SSI rates using PVI irrigation compared to using intrawound antibiotics. Therefore, given the clear differential effects we report here on osteoblast viability, it would appear pertinent for clinicians to favour the practice of intrawound Vancomycin or Gentamicin application over 0.35% PVI wound irrigation. Furthermore, although the combination of Vancomycin intrawound with PVI wound irrigation was recently reported to provide a commendable 50% reduction in SSI



225 rates in spinal surgery <sup>22</sup>, our data suggests this practice should be approached  
226 cautiously. Such caution may be particularly warranted for paediatric patients  
227 undergoing long fusion surgical procedures (4 levels of more), with high non-union  
228 rates <sup>30,31,32</sup>, where any gains in reducing SSI rates with PVI alone (or in combination  
229 with Vancomycin) may be offset by impaired bone healing.

231 It is important to stress the limitations of this study. It is an *in vitro* study, and  
232 although the human osteoblasts are primary cells they do not originate from spinal  
233 bone tissue. Furthermore, such *in vitro* studies cannot fully replicate the complexity  
234 of the *in vivo* environment, where for example pluripotent stem cells may migrate to  
235 the site of surgery and aid bone healing and fusion. Nevertheless, our data suggests  
236 a clear differential between the effects of Vancomycin and Gentamicin antibiotics  
237 compared to PVI on osteoblast proliferation and function.

239 In summary, at concentrations advocated for the reduction of SSIs in spinal surgery  
240 Vancomycin and Gentamicin had no sustained effect on osteoblast proliferation, or  
241 the formation of mineralised bone. We suggest therefore that in contrast to PVI  
242 wound irrigation, the intrawound application of the either Vancomycin or Gentamicin  
243 in spinal surgery would be unlikely to impair bone healing.

244 **Abbreviations**

245

246 **PVI** Povidone-Iodine

247 **PBS** Phosphate buffered saline

248 **MTS** 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

249

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## Figure legends

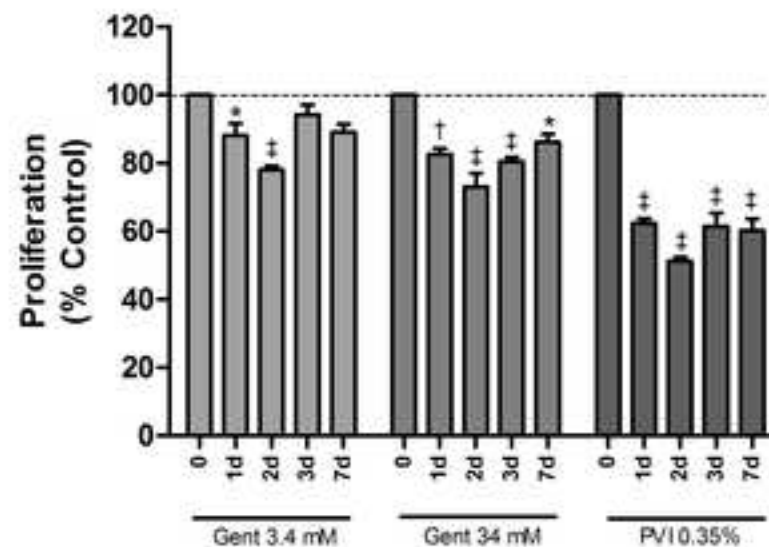
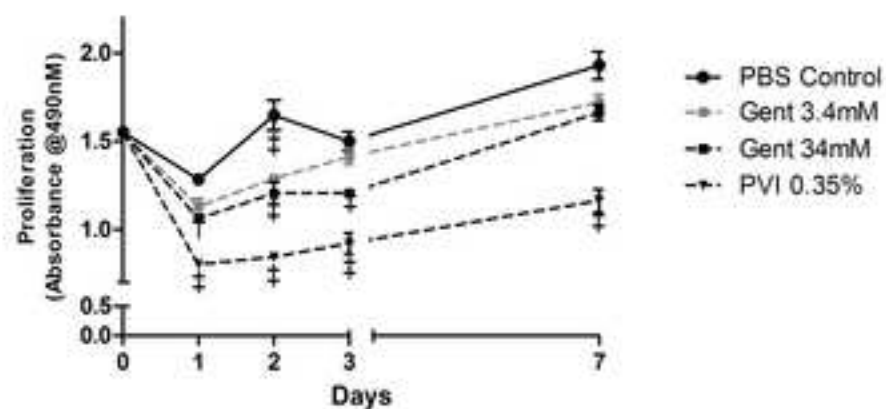
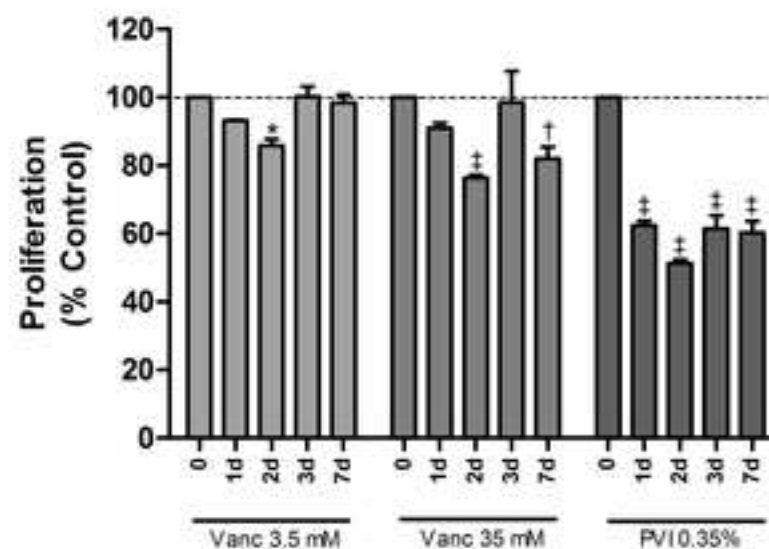
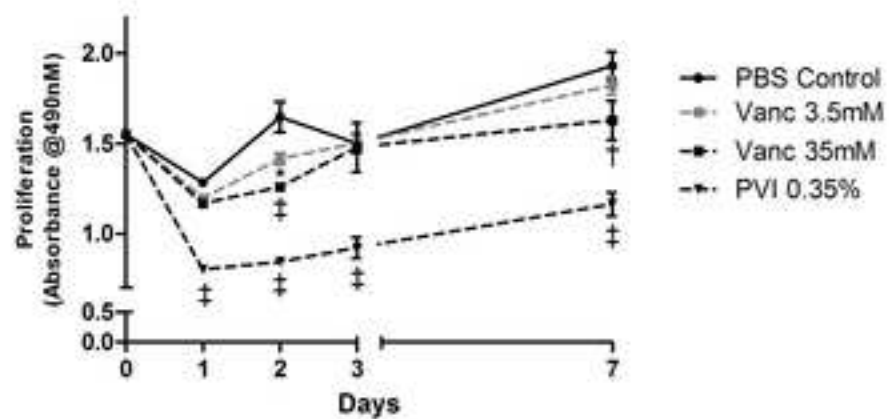
**Figure 1. The effect of acute exposure to antibacterial intrawound reagents on primary human osteoblast proliferation.** (A) Osteoblasts were stimulated for 20 min with either PBS control, Gentamicin (34 mM or 3.4 mM) or stimulated for 3 min with 0.35% PVI. (B) Osteoblasts were stimulated for 20 min with either PBS control, Vancomycin (35 mM or 3.5 mM) or stimulated for 3 min with 0.35% PVI. Osteoblast proliferation was measured by MTS assay (absorbance at 490 nm) at time 0, day 1, 3, 4 and day 7. Bars represent mean  $\pm$  SEM (n=3), with \*= $P<0.05$ , †= $P<0.01$ , ‡= $P<0.001$ , representing values significantly different to PBS control.

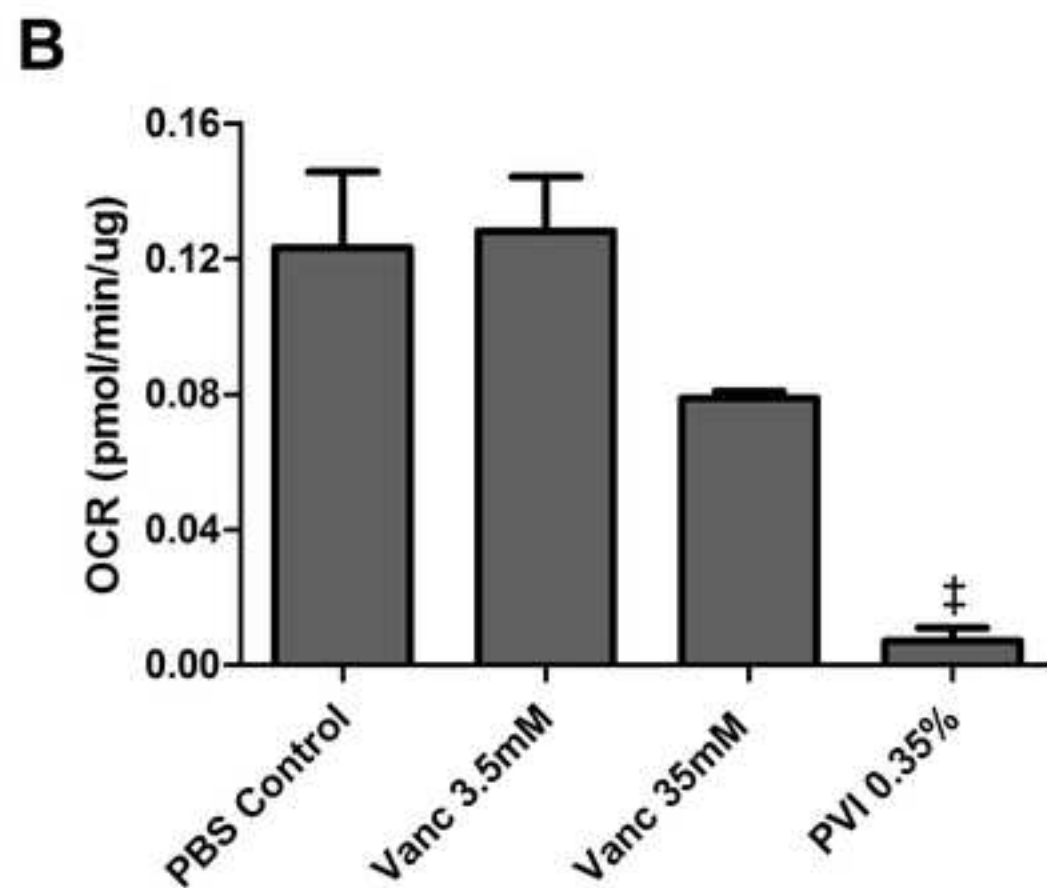
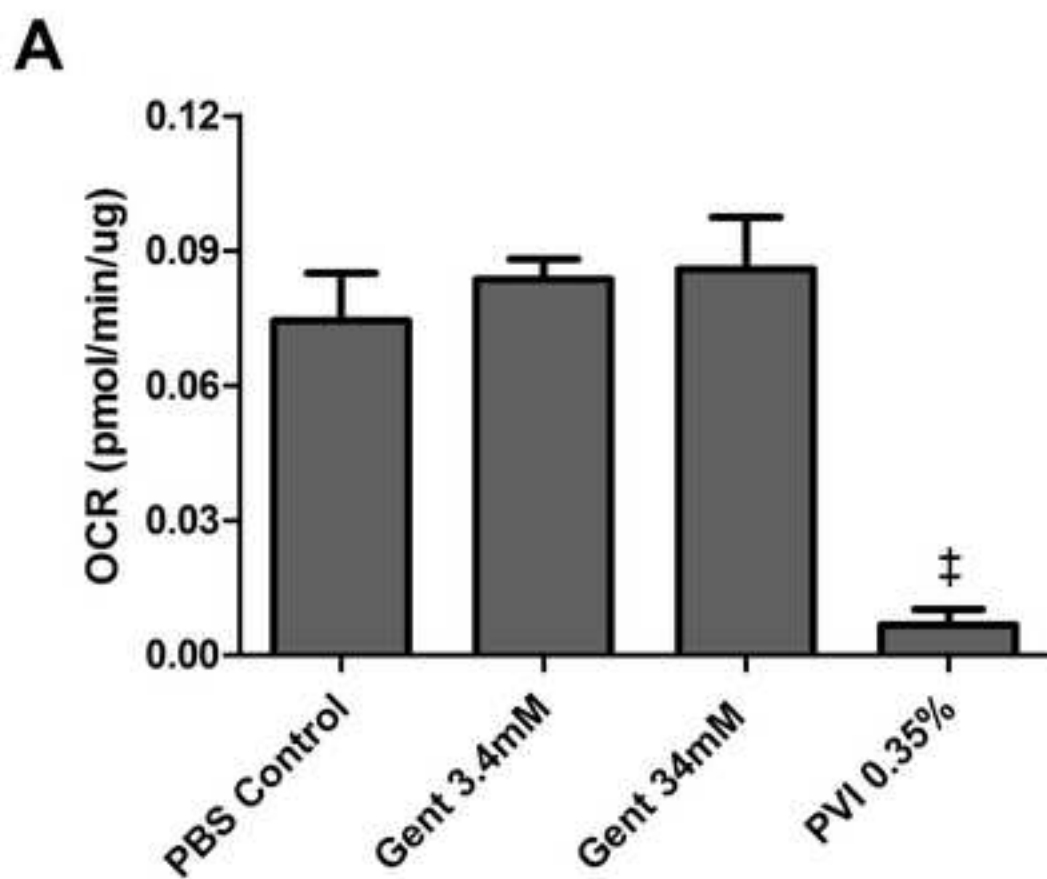
**Figure 2. Metabolic activity of primary human osteoblast following acute exposure to antibiotic intrawound reagents.** (A) Oxygen consumption rate (OCR) of osteoblasts 24 h after 20 min exposure to either Gentamicin (34 mM or 3.4 mM) or 3 min exposure to 0.35% PVI. (B) OCR of osteoblasts after 20 min exposure to either Vancomycin (35 mM or 3.5 mM) or 3 min exposure to 0.35% PVI. Bars represent mean  $\pm$  SEM (n=3), with ‡= $P<0.001$ , representing values significantly different to PBS control.

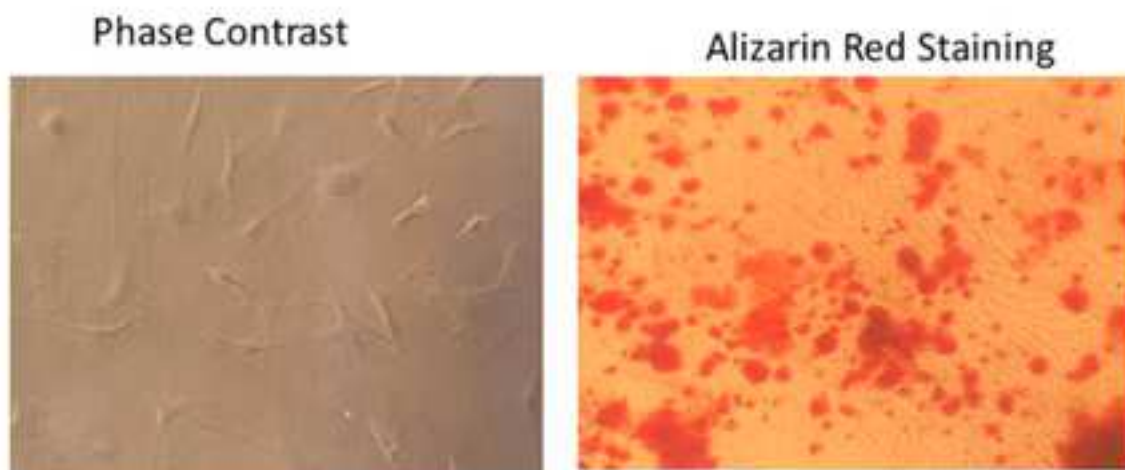
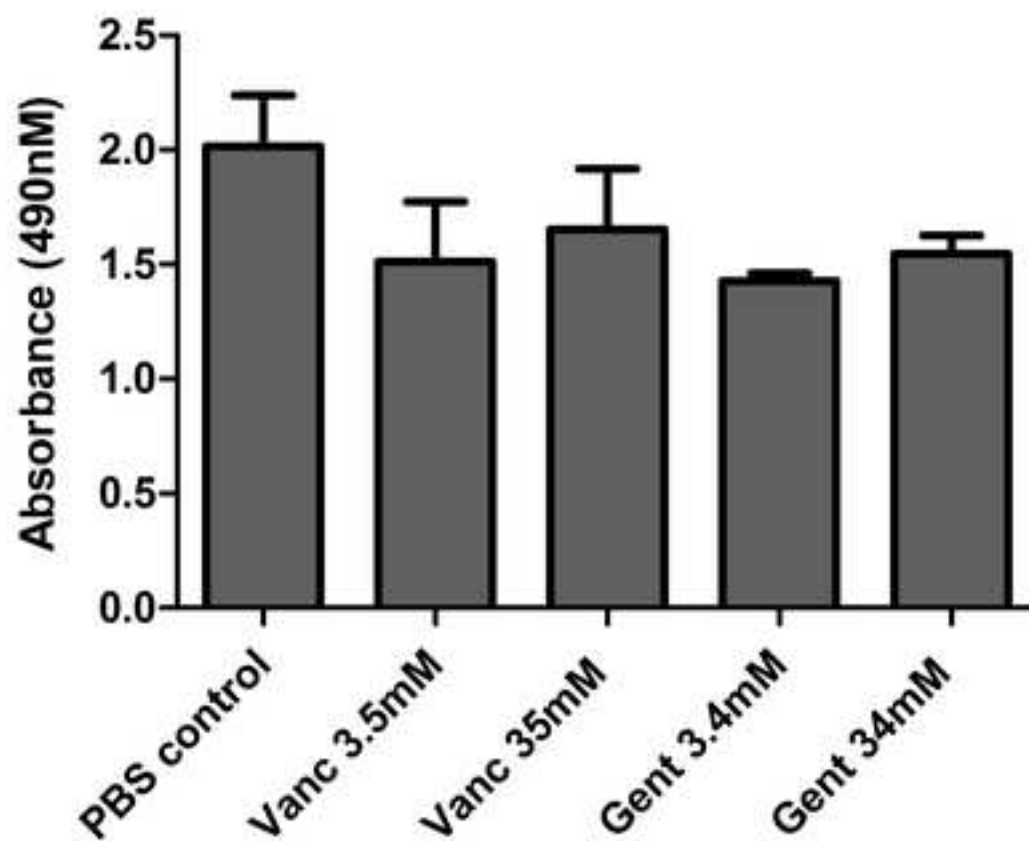
**Figure 3. Primary human osteoblast mineralisation following acute exposure to antibiotic intrawound reagents.** (A) Representative microscopic images (x20 magnification) of phase contrast of primary human osteoblasts, and Alizarin red stained mineralised bony nodules after 28 days post fully confluent. Primary human osteoblasts were cultured out bone chips in differentiation media. (B) Quantification by absorptiometry (at 490 nm) of Alizarin red stained mineralised bone

nodules in osteoblast 14 days after acute 20 min exposure to either Gentamicin (34 mM or 3.4 mM), Vancomycin (35 mM or 3.5 mM) or PBS media control. Bars represent mean  $\pm$  SEM (n=3). (C) Representative microscopic images showing images of Alizarin red stained mineralised bone nodules 14 days after exposure to Gentamicin and Vancomycin antibiotics.



**A****B**



**A****B****C**